Unique responses of the avian macrophage to different species of *Eimeria*

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Abstract

Coccidiosis is recognized as the major parasitic disease of poultry and is caused by the apicomplexan protozoa *Eimeria*. Increasing evidence shows the complexity of the host immune response to *Eimeria* and microarray technology presents a powerful tool for the study of such an intricate biological process. Using an avian macrophage microarray containing 4906 unique gene elements, we identified important host genes whose expression changed following infection of macrophages with sporozoites of *Eimeria tenella* (ET), *Eimeria acervulina* (EA), and *Eimeria maxima* (EM). This approach enabled us to identify a common core of 25 genetic elements whose transcriptional expression is induced or repressed by exposure to *Eimeria* sporozoites and to identify additional transcription patterns unique to each individual *Eimeria* species. Besides inducing the expression of IL-1β, IL-6, and IL-18 and repressing the expression of IL-16, *Eimeria* treated macrophages were commonly found to induce the expression of the CCL chemokine family members macrophage inflammatory protein (MIP)-1β (CCLi1), K203 (CCLi3), and ah221 (CCLi7). However, the CXCL chemokine K60 (CXCLi1) was found to be induced by macrophage exposure to *E. tenella* but was repressed upon macrophage exposure to *E. maxima* and *E. acervulina*. Fundamental analysis of avian chemokine and cytokine expression patterns offers insight into the unique avian immunological responses to these related but biologically unique pathogens.
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1. Introduction

Coccidiosis is recognized as the major parasitic disease of poultry and is caused by the apicomplexan protozoa *Eimeria*. Coccidiosis seriously impairs the growth and feed utilization of infected birds resulting in loss of productivity and it inflicts economic losses in excess of US $3 billion annually to the world poultry industry (Shirley et al., 2004; Williams, 1999). Conventional disease control strategies rely heavily on chemoprophylaxis and to a certain extent on live vaccines (Dalloul and Lillehoj, 2005). Increasing regulations and bans on the use of anticoccidial drugs coupled with the associated costs for developing new drugs and live vaccines has stimulated the need for developing novel approaches and alternative control strategies for coccidiosis. However, such new approaches will only be realized after a systematic and detailed analysis of host–parasite interactions at the molecular and cellular levels are completed. In particular, fundamental knowledge of the basic immunobiology from initial parasite invasion to intracellular development and ultimate elimination from the host is very limited. Increasing evidence demonstrates the complexity of the host immune responses to *Eimeria*. Additional basic research is needed to ascertain the detailed immunological and physiological processes mediating protective immunity.

Chickens have evolved a sophisticated immune system where macrophages play significant roles in both the innate and acquired immune responses. As in mammals, avian macrophages act as antigen presenting cells for B and T lymphocytes and stimulate the activation of other immune

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and non-immune cells through the release of various lymphokines and cytokines (Dalloul and Lillehoj, 2006). Furthermore, macrophages are responsible for the clearance and destruction of both intracellular and extracellular pathogens through phagocytosis. In coccidiosis, chicken macrophages are involved in different phases of the host immune response to *Eimeria* (Dalloul and Lillehoj, 2006). In *Eimeria tenella* (ET)-immune chickens, macrophages and other leukocytes infiltrate the ceca more rapidly than in naïve chickens (Vervelde et al., 1996). Also, macrophages pretreated with the culture supernatants of Con A-stimulated spleen cells or T cells exert cytostatic effects on the growth of *E. tenella* sporozoites (Dimier et al., 1998).

The microarray has become a powerful tool for the study of immune system function. Although a variety of large-scale commercial arrays are available for human and other mammalian species, there are few such tools available for agricultural species. In the avian, a small number of low-density and high-density cDNA based microarrays have been developed (Cogburn et al., 2004; Koskela et al., 2003; Min et al., 2003; Morgan et al., 2001; Neiman et al., 2003). More recently, a consortium of research groups has developed a comprehensive 13,000 element chicken cDNA microarray (http://www.fhcrc.org/shared_resources/genomics/chicken_13k.pdf) and a commercially available whole genome chicken oligonucleotide array (Affymetrix Corp., Sunnyvale, CA) has been developed for use by the avian research community.

To aid in studies of the avian innate immune response we have recently constructed a 4906 element (14,718 spot) avian macrophage-specific cDNA microarray (AMM). This array has been used to examine the transcriptional response of avian macrophages to Gram-negative bacteria (*Escherichia coli*) and their cell wall components (LPS) and has specifically been used to evaluate the contribution of the TLR pathway to this response (Bliss et al., 2005). This approach has enabled us to significantly enhance our understanding of the innate immune response mediated by the avian macrophage in response to bacteria. In the current study, the AMM was used to elucidate the avian macrophage's transcriptional response to three related but biologically distinct avian protozoan pathogens: *Eimeria acervulina* (EA), *Eimeria maxima* (EM), and *E. tenella*. This approach enabled us to identify common genetic elements whose transcriptional expression is induced by exposure to *Eimeria* sporozoites and to identify transcription patterns unique to an individual *Eimeria* species. Analysis of avian chemokine and cytokine expression patterns offers insight into the avian immunological responses to these related but biologically unique pathogens.

2. Materials and methods

2.1. Macrophage cell culture and Eimeria species

Avian macrophage HTC cells (Rath et al., 2003) were grown overnight in RPMI 1640-complete (Sigma, St. Louis, MO) containing 10% FCS (HyClone, Logan, UT), 2.0 mM glutamine, 1.0 mM sodium pyruvate, 0.5 μM 2-mercaptoethanol, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 10 mM HEPES (Sigma), pH 7.3 at 40 °C in 5% CO_2. HTC cells were seeded at 5×10^6 cells/ml in 24-well plates and exposed to the same concentration of freshly prepared sporozoites of *E. acervulina*, *E. maxima*, or *E. tenella*, for 0, 4, 18, and 48 h (triplicate wells each). Cells were then washed, triplicates pooled, and total macrophage RNA prepared using TRIzol reagent (Invitrogen, Gaithersburg, MD).

2.2. RNA preparation and microarray hybridization

The AMM was constructed and spotting quality was evaluated as previously described (Bliss et al., 2005). Purified PCR products (∼150 ng/μl) were spotted in triplicate (14,718 total spots) onto Telechem SuperAmine slides (Telechem International Inc., Sunnyvale, CA) using a GeneMachines Omnigrid Accent spotter (GeneMachines, San Carlos, CA). Slide design is included in supplemental data on a web database at http://www.aviangenomics.udel.edu. PolyA(+) RNA was purified from TRIzol-prepared total RNA using the Ambion MicroPoly(A) Purist Purification Kit according to the manufacturer's instructions (Ambion Inc., Austin, TX). RNA concentrations were determined spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE).

PolyA(+) macrophage RNA (1.5 μg) was transcribed into cDNA and fluorescently-labeled with AlexaFluor 555 or AlexaFluor 647 (Molecular Probes) through the use of the Amino Allyl cDNA Labeling Kit (Ambion Inc.). Concentration and labeling efficiencies of cDNA were determined spectrophotometrically. For hybridization, a circular loop design (Townsend, 2003) was employed for the four time points (0, 4, 18, and 48 h) within each *Eimeria* treatment. Two color microarray hybridizations (65 μl) were performed using HybIt hybridization buffer (Telechem International Inc.) in Mica hybridization chambers (GeneMachines) at 50 °C overnight. After hybridization, slides were rinsed in 0.5 × SSC, 0.01% SDS at room temperature and then washed for 15 min in 0.2 × SSC, 0.2% SDS at 50 °C, three times for 1 min in 0.2 × SSC at room temperature, and finally three times for 1 min in water at room temperature. Washed slides were scanned with an ArrayWoRx scanner (Applied Precision, Issaquah, WA) using Cy3 and Cy5 filters.

2.3. Microarray data acquisition, processing, and analysis

Spot and background intensities were acquired using SoftWoRx tracker (Applied Precision) and data analysis was performed using GeneSpring v6.1 (Silicon Genetics, Redwood City, CA). Background intensity was determined using the GeneSpring cell method. Abnormal spots (dust, bubbles) and spots with intensities not greater than background plus two standard deviations were flagged. Elements that were not represented by at least two replicate spots (not flagged as abnormal or low signal) on every slide used in the experiment were removed from further analysis. On each slide, spot intensities were normalized to the median background-subtracted spot intensity of that slide and then to the control (0 h) channel value so that fold change from control could be determined. Those elements exhibiting

>2-fold changes in signal intensity during at least one time point were analyzed by ANOVA using the Benjamini and Hochberg (1995) false discovery rate (FDR) multiple testing correction with a p-value of <0.001 to determine which biologically significant changes were also statistically significant. Genetic elements displaying common patterns of expression and elements of specific cytokine and chemokine functional classes were clustered by Gene Tree analysis within the GeneSpring software package. In compliance with the MIAME guidelines, information on the AMM and additional supplemental data are available on a web database at http://www.aviangenomics.udel.edu and at the NCBI GenBank Gene Expression Omnibus (GEO) repository, series accession number GSE3723.

2.4. Quantitative real-time RT-PCR (Q-RT-PCR)

Oligonucleotide primers for cytokines, chemokines and GAPDH quantitative RT-PCR are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA, isolated using TRIzol reagent from cultured macrophages as described above (same RNA samples used in microarray experiments), with the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene). Standard curves for the cytokines, chemokines and GAPDH were generated using Q-gene program (Muller et al., 2002). Each experiment was performed in triplicate and the $\log_{10}$ diluted standard RNA was used to generate standard curves. To normalize RNA levels between samples within an experiment, the mean threshold cycle (C_t) value for the cytokines, chemokines and GAPDH products were calculated by pooling values from all samples in that experiment. Transcript levels were normalized to those of GAPDH using the Q-gene program (Muller et al., 2002).

3. Results

3.1. Gene expression in Eimeria-stimulated avian HTC macrophage cell culture

Avian macrophage HTC cells were exposed to *E. acervulina*, *E. maxima*, or *E. tenella* sporozoites for 0, 4, 18, and 48 h.

Table 1
Oligonucleotide primers for cytokines, chemokines and GAPDH quantitative RT-PCR

RNA target	Primer sequences	PCR product size (bp)	Accession no.
GAPDH			
Forward	5′-GGTGGTGCTAAGCGTGTTAT-3′	264	K01458
Reverse	5′-ACCTCTGTCATCTCTCCACA-3′		
IFN-γ			
Forward	5′-AGCTGACGGTGGACCTATTATT-3′	259	Y07922
Reverse	5′-GGCTTTGCGCTGGATTC-3′		
IL-1β			
Forward	5′-TGGGCATCAAGGGCTACA-3′	244	Y15006
Reverse	5′-TCGGGTTGGTTGGTGATG-3′		
IL-6			
Forward	5′-CAAGGTGACGGAGGAGGAC-3′	254	AJ309540
Reverse	5′-TGGCGAGGAGGGATTTCT-3′		
IL-8			
Forward	5′-GGCTTGCTAGGGGAAATGA-3′	200	AJ009800
Reverse	5′-AGCTGACTCTGACTAGGAAACTGT-3′		
IL-18			
Forward	5′-GGAATGCGATGCCTTTTG-3′	264	AJ277865
Reverse	5′-ATTTTCCCATGCTCTTTCTCA-3′		
K60			
Forward	5′-ATTTCCTCCTGCCTCCTACA-3′	228	AF277660
Reverse	5′-GTGACTGGCAAAAATGACTCC-3′		
K203			
Forward	5′-ACCACGAGCTCCTGACACA-3′	300	Y18692
Reverse	5′-TTAAATGCCCTCCCTACCAC-3′		
MIP-1β			
Forward	5′-GTGCCCTCATGCTGGTGT-3′	285	L34553
Reverse	5′-GGTTGGATGCGGATTATTTC-3′		
iNOS			
Forward	5′-TGGGTGGAAGCCGAAATA-3′	241	U46504
Reverse	5′-GTACCAGCCGTTGAAAGGAC-3′		
Ah221			
Forward	5′-AAAACTGACCCTATCCTGCTCTCG-3′	256	XM415781
Reverse	5′-AGGATCGGGGTTGGAACTTGGTGA-3′		

Table 2
Statistical analysis of AMM data quality and elements displaying significant (>2-fold) changes in expression

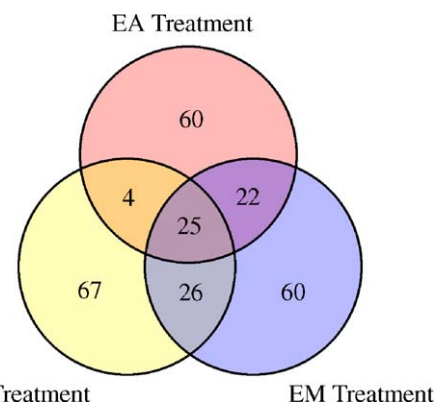|	
Elements on the AMM	4906
Elements with high-quality replicate data	3140
Elements exhibiting >2-fold change in EA treatment	288
Statistically significant >2-fold changes in EA treatment	111
Elements exhibiting >2-fold change in EM treatment	262
Statistically significant >2-fold changes in EM treatment	134
Elements exhibiting >2-fold change in ET treatment	282
Statistically significant >2-fold changes in ET treatment	122
Total number of unique elements exhibiting significant expression changes	265

Fig. 1. AMM elements exhibiting a significant change in expression. Avian macrophage HTC cells were exposed to *E. acervulina* (EA), *E. maxima* (EM), or *E. tenella* (ET) sporozoites for 0, 4, 18, and 48 h. PolyA(+) mRNA from these cells was purified, fluorescently-labeled, and hybridized to AMM slides. The number of elements exhibiting a statistically significant, >2-fold change over control levels following stimulation by *E. acervulina*, *E. maxima*, or *E. tenella* is indicated.

PolyA(+) mRNA from these cells was purified, fluorescently-labeled, and hybridized to AMM slides containing 4906 unique macrophage expressed elements (spotted in triplicate). Sixty-four percent (3140) of the elements on the array produced high-quality replicate data for at least two spots in all 24 labelings (Table 2). Only these elements were used for further statistical analysis. Of these elements, 8.3–9.0% (262–288) showed >2-fold expression changes within each of the three treatments with nearly half of these (111–134 or 3.5–4.3%) being statistically significant (Table 2).

A comparison of the elements exhibiting significant expression changes in each treatment found that 25 elements on the array were commonly regulated in all three treatments (Fig. 1). This represents a set of core response genes that are induced or repressed in response to *Eimeria* exposure. The majority of

these 25 core response elements were induced by all three treatments, with only 5 elements being commonly repressed (Fig. 2). The core elements include several important immune effector genes, such as two members of the CCL macrophage inflammatory protein (MIP) family, chemokine ah221 (CCLi7) and MIP-1β (CCLi1), as well as seven elements of unknown function as determined by BLAST homology to the NCBI nucleotide database and the TIGR *Gallus gallus* Gene Index.
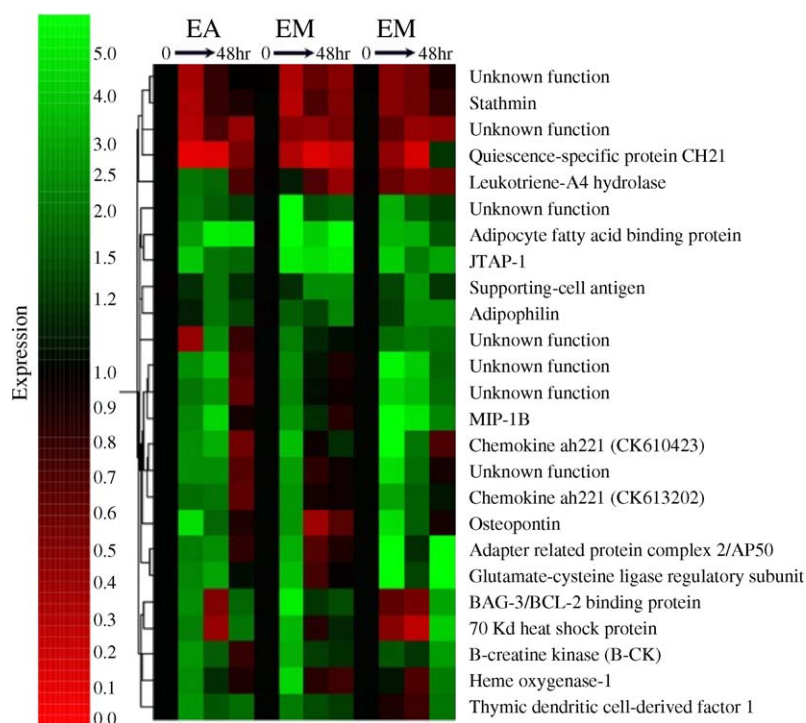

Fig. 2. Gene tree of the 25 core AMM elements commonly expressed by HTC macrophages following exposure to *E. acervulina* (EA), *E. maxima* (EM), or *E. tenella* (ET) sporozoites for 0, 4, 18, and 48 h. PolyA(+) mRNA from TRIzol prepared total RNA of these cells was purified, fluorescently-labeled, and hybridized to AMM slides.

3.2. Differential gene expression following stimulation with Eimeria sp.

A comparison of elements exhibiting significant expression changes in each treatment found that each strain of *Eimeria* induced significant (>2-fold) expression changes in a unique set of approximately 60 elements (Fig. 1). This comparison also showed that treatments with *E. acervulina* and *E. tenella* induced changes in almost no common genes (4) with the exception of the 25 core elements. However, *E. acervulina* and *E. maxima* commonly induced expression changes in a set of 22 genes uniquely different from the set of 26 genes exhibiting expression changes

Table 3
The 10 most highly induced and repressed annotated genes following each treatment, as determined by microarray

Induced elements			Repressed elements		
Clone ID	Fold change	Function	Clone ID	Fold change	Function
E. acervulina treatment					
Controlb_O13	5.46	Adipocyte fatty acid binding protein (AFABP)	IFNg_C24	10.28	Quiescence-specific protein precursor; CH21
Control_C08	5.03	Complement subcomponent C1q chain C precursor	IFNb_B10	3.61	Olfactory receptor protein
Controle_A03	4.88	Osteopontin	pmp1c.pk007.n14	3.13	Stathmin
IFNk_L14	4.55	Macrophage inflammatory protein 1-beta (CCLi1))	IFNe_K10	3.02	Protein translation factor SUI1 homolog (Sui1iso1)
pmp1c.pk003.e04	4.53	Collagenase 3	LPSc_I18	2.96	Sideroflexin 1 (SFXN1)
Control_B23	4.46	RSFR; ribonuclease A/angiogenin	IFNh_F12	2.95	N-myc downstream regulated gene 1 (NDRG1)
IFNm_E24	4.07	v-Jun transformation assoc. target protein (JTAP-1)	LPSf_H04	2.74	Annexin II
LPSc_A07	3.65	Interleukin-18 (IL-18)	LPSf_O06	2.72	Proline-rich protein 2 (B4-2 protein)
Controlb_J06	3.61	Prepro-cathepsin D	IFNd_N10	2.67	Spermidine/spermine N1-acetyltransferase
LPSl_L04	3.56	Sorting nexin 17	LPSf_D19	2.59	MAX binding protein MNT / MYC antagonist MNT
E. maxima treatment					
Controlb_O13	11.49	Adipocyte fatty acid binding protein (AFABP)	IFNg_C24	8.77	Quiescence-specific protein precursor; CH21
IFNi_J24	7.79	Transcription factor ATF-3; LRG-21	pmp1c.pk004.k20	3.76	Lipopolysaccharide binding protein (LBP)
IFNm_L11	6.34[a]	Serine protease HTRA1	pmp1c.pk007.n14	3.45	Stathmin
LPSf_P08	5.47	BAG-3; Bcl-2-binding protein	LPSk_C16	3.40	Interleukin 1 receptor type II
IFNm_E24	5.47	v-Jun transformation assoc. target protein (JTAP-1)	LPSi_D05	3.33	Thymidine kinase (TK)
LPSf_O11	5.15	Virion-associated nuclear-shuttling protein; p54	LPSf_E01	3.27	Avidin
Controld_K20	4.62	Heme oxygenase-1	LPSb_J23	3.21	gp91-phox; cytochrome b-245, beta polypeptide
IFNk_A23	4.53[a]	Prepro-cathepsin D	IFNb_B10	3.15	Olfactory receptor protein
LPSk_A24	3.81	Glutamate-cysteine ligase regulatory subunit	IFNe_O03	2.99	Scavenger receptor MARCO
IFNd_E10	3.72	Chemokine ah221 (CCLi7)	IFNh_H07	2.86	FK506-binding protein
E. tenella treatment					
LPSk_A24	8.53	Glutamate-cysteine ligase regulatory subunit	IFNg_C24	6.45	Quiescence-specific protein precursor; CH21
IFNc_A17	8.36[a]	CXCLi1 chemokine K60	IFNe_O03	4.65	Scavenger receptor MARCO
LPSk_M12	8.09	Adaptor-related protein complex 2; AP50	Controlb_P15	4.35	c-fos proto-oncogene
IFNd_E10	7.09	Chemokine ah22 (CCLi7)1	LPSl_H10	4.17	70 kd heat shock protein
LPSe_C09	6.71[a]	Serine protease HTRA1	LPSb_D08	4.07	NADH2 dehydrogenase (ubiquinone) chain 5
IFNk_L14	6.21	Macrophage inflammatory protein 1-beta (CCLi1)	LPSm_B01	3.48	E3 protein
Controle_A03	4.81	Osteopontin	Controlb_E01	3.13	Gamma-parvin
LPSe_E09	4.59	K123 protein	LPSb_H07	2.82	Heat shock cognate 70
IFNi_J24	4.49	Cyclic-AMP-dependent transcription factor ATF-3	Controld_L19	2.62	Thymosin beta 4
IFNm_E24	4.25	JTAP-1	IFNj_C08	2.47	Death-associated protein 1 (DAP-1)

[a] Fold change is an average of the fold changes from multiple elements on the array representing that gene

when macrophages were exposed to *E. tenella* and *E. maxima* (Fig. 1).

The 10 most highly induced and repressed annotated genes following each treatment, as determined by microarray, are listed in Table 3. Many important immune effector genes appear among these most highly induced elements lists, including complement component C1q, MIP-1β (CCLi1), and the chemokines K60 (CXCLi1) and ah221 (CCLi7). While some genes are highly induced following two of the three treatments (AFABP, MIP-1β, and JTAP-1), no common induced genes are found in all three treatments. However, the quiescence specific protein precursor, CH21, is among the most highly repressed in all three treatments, being the most highly repressed following *E. acervulina* and *E. tenella* exposure and the second most highly repressed following *E. maxima* exposure, where an element of unknown function was highest repressed (>11-fold).

3.3. Avian cytokine and chemokine gene expression patterns

The AMM contains a significant number of avian cytokine and chemokine genes. Many of these elements demonstrated significant expression changes following stimulation with different *Eimeria* species. A set of 10 of these genes is shown in Fig. 3. IL-1β and MIP-1β (CCLi1) show the most dramatic and consistent expression inductions, while IL-16 expression was consistently repressed (Fig. 3). Several of the cytokine/chemokine elements also show differential expression following treatment with different *Eimeria* species, including chemokine K60

(CXCLi1), which is highly induced at 48 h in *E. tenella* exposed macrophages and is repressed at 48 h in *E. acervulina* and *E. maxima* exposed macrophages.

The expression patterns of four of these 10 genes (K60, ah221, IL-18, and MIP-1β) were confirmed by quantitative real-time RT-PCR (Q-RT-PCR) (Table 4, Fig. 4). Although the amplitude of these changes was generally found to be higher by Q-RT-PCR than by array analysis, the temporal regulation patterns found by the two methods were nearly identical.

4. Discussion

Eimeria parasites are ubiquitous pathogens and the causative agents of poultry coccidiosis, one of the most costly endemic diseases to the poultry industry worldwide. Three species, *E. acervulina*, *E. maxima*, and *E. tenella*, are the most commonly encountered in the field with each infecting a specific intestinal site. Infections, when not deadly, induce protective immunity against subsequent challenges; however, such immunity remains confined to homologous species with no cross-species protection (Dalloul and Lillehoj, 2006). Among the three, *E. maxima* is characterized by high immunogenicity where priming infection with only a few oocysts induces full protective immunity to subsequent homologous challenge. Conversely, far more *E. acervulina* and *E. tenella* oocysts are required to induce comparable levels of protective immunity. For these reasons, there is a pressing need to elucidate the fundamental similarities as well as differences in the immune responses induced by these three related but distinctly unique pathogens. Identification of
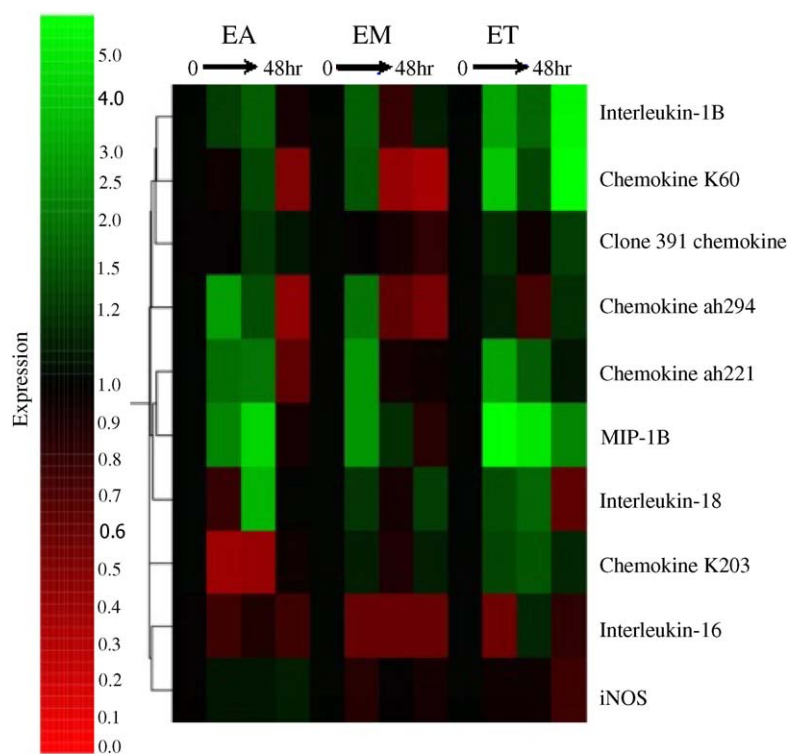

Fig. 3. Gene tree of the 10 most highly induced and repressed cytokine/chemokine genes following each treatment, as determined by microarray. Avian macrophage HTC cells were exposed to *E. acervulina* (EA), *E. maxima* (EM), or *E. tenella* (ET) sporozoites for 0, 4, 18, and 48 h. PolyA(+) mRNA from these cells was purified, fluorescently-labeled, and hybridized to AMM slides.

Table 4
Expression changes of selected genes in response to three species of *Eimeria*

Gene	Sporozoite treatment			Measured by
	E. acervulina	*E. maxima*	*E. tenella*	
Interleukin-1β	++	++	++	Q-PCR
Interleukin-6	+	++	+	QPCR
Interleukin-18	++	+	+	Array and Q-PCR
Chemokine K60	−	−	++	Array and Q-PCR
Chemokine K203	++	++	++	Array and Q-PCR
Chemokine ah221	+	+	+	Array
MIP-1β	+	+	++	Array
Interferon-γ	-	−	++	Q-PCR
iNOS	+	++	++	Q-PCR

'+', Induction; '−';repression; 2–5-fold = 1 signal, >5-fold = 2 signals.

the early host responses at the gene transcription level provides a molecular immune profile of the events that occur during and immediately following infection with *Eimeria* sp. In this study, we stimulated avian macrophages in vitro with three *Eimeria* sp. sporozoites, the invasive stages of the parasite, and by employing a recently developed avian macrophage microarray, we identified immunity-related genes that were either induced or repressed following exposure.

Determining which macrophage genes are transcribed during the early stages of *Eimeria* sp. infection can be used to determine the molecular pathogenesis of coccidiosis. By infecting the avian HTC macrophage cell line, common as well as species-specific host responses were identified. This study also shows that early macrophage activation events induced by individual species of *Eimeria* appears to correlate with the number of genes and the overall magnitude of the transcriptional response elicited by each individual species. Indeed, almost the same

number of elements changed in response to the three species and individual species uniquely induced expression changes in a similar number of elements. A set of core response elements has been identified comprising 25 genes, including many immune-related genes, while 60–67 elements were uniquely induced or repressed by individual species. Such differential responses may be attributed to the species-specific immunity induced by each *Eimeria* sp. and a deeper look into the functional aspects of those elements could prove critical in shedding some light on the lack of cross-species protection. Further characterization of both sets of elements would help elucidate the pathogenicity and/or immunogenicity of each species leading to better recombinant vaccine design and control strategies.

The majority of the 25 core response elements were induced by all three treatments and they included several important immune effector genes, such as the chemokines ah221 (CCLi7) and MIP-1β (CCLI1), and osteopontin. While many elements

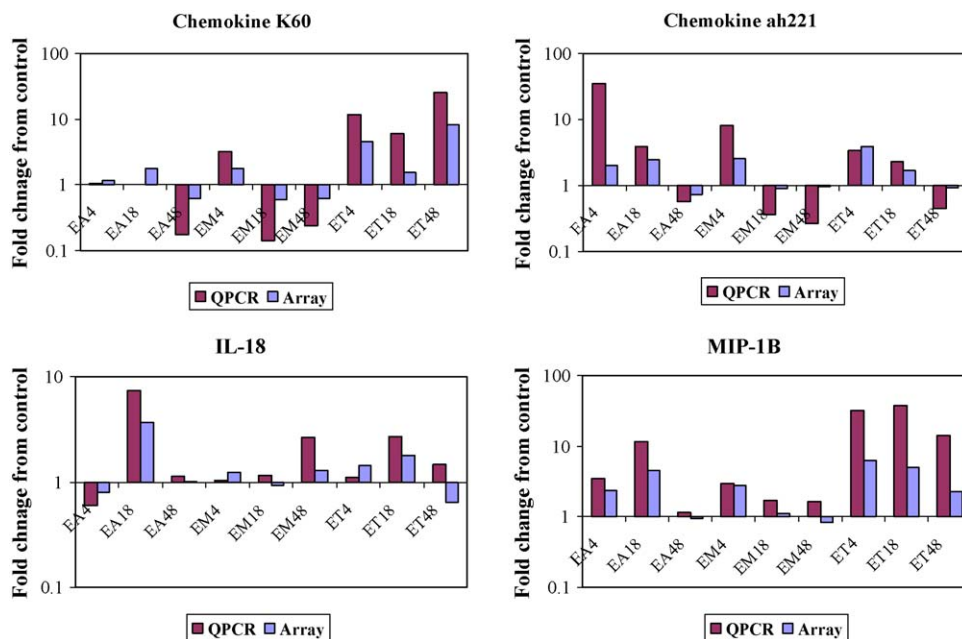Fig. 4. Confirmation of microarray results by quantitative real-time reverse transcription PCR (Q-RT-PCR). Avian macrophage HTC cells were exposed to *E. acervulina* (EA), *E. maxima* (EM), or *E. tenella* (ET) sporozoites for 0, 4, 18, and 48 h. The expression patterns of four immune-related genes, K60, ah221, IL-18, and macrophage inflammatory protein (MIP)-1β, characterized by microarray analysis were confirmed by Q-RT-PCR.

were highly induced following two of the three treatments, none of the 25 core elements fell among the top 10 induced genes; but the quiescence-specific protein precursor CH21 was among the most highly repressed genetic elements in all three treatments. As measured by Q-RT-PCR (Table 4), the proinflammatory cytokine IL-1β was highly induced (>5-fold) by the three species (Fig. 3). IL-1β is secreted by macrophages and other cells upon activation by stimuli (Rodenburg et al., 1998), which in turn upregulates the production of other chemokines like MIP-1β, K203, and ah221, and cytokines like osteopontin, thereby amplifying the immune response. MIP-1β and K203 belong to the CC chemokine family, normally involved in the recruitment of macrophages and they were both upregulated in all three treatments. Using IFN-γ stimulated macrophages (HD11), Laurent et al. (2001) observed similar results suggesting that macrophages are the main effector inflammatory cells at *Eimeria* infection sites. Osteopontin has been described as an important component of early cellular immune responses (Patarca et al., 1993). It is known to directly induce chemotaxis and to indirectly facilitate macrophage migration to other chemoattractants, and has been characterized as an early protein expressed by activated macrophages and natural killer cells (O'Regan et al., 2000). Osteopontin enhances T helper 1 (Th1) and inhibits Th2 cytokine expression. In mice, it directly induces macrophages to produce IL-12, and inhibits IL-10 expression by LPS-stimulated macrophages (Ashkar et al., 2000). In chickens, *Eimeria* infections induce Th1 immune responses (Dalloul and Lillehoj, 2005) and this observation further indicates such a premise. The paradigm of Th1/Th2 cytokine polarization suggests that early expression of Th1 cytokines is critical to a protective host response against intracellular infection (Abbas et al., 1996) like coccidiosis. Therefore, factors (including antigens) augmenting Th1, and inhibiting Th2, cytokine expression might function as powerful modulators of cell-mediated immunity (CMI), the main effector branch of the bird's immune system against coccidiosis (Dalloul and Lillehoj, 2006; Lillehoj et al., 2004).

Conversely, other elements like IL-16 (Fig. 3) and quiescence-specific protein (Table 3) were consistently repressed in all three treatments. Quiescence-specific protein is a secreted 20 kDa molecule belonging to the Lipocalin protein family and is among the most prevalent proteins present in quiescent chicken heart mesenchymal (CHM) cells (Bédard et al., 1987) and chick embryo fibroblasts (CEF) (Mao et al., 1993). By contrast, this protein is virtually absent in actively dividing cells, as is the case with the HTC cells used in this study. However, during intracellular infection (herpesvirus) of CEF, Morgan et al. (2001) observed a high level of expression of the gene, suggesting that the virus inhibits cell cycle progression while allowing those cells to accumulate factors needed for its own replication. Interestingly, some cytokine/chemokine elements were differentially expressed following treatment with different *Eimeria* species, including K60 (CXCLi1) and IFN-γ. K60 was highly induced at 48 h in *E. tenella* exposed macrophages but repressed at 48 h in *E. acervulina* and *E. maxima* treated macrophages. IFN-γ was also highly expressed in *E. tenella* treatment but not in the other two. In vivo K60 transcripts levels have been shown

to remain unchanged or increase slightly compared to levels of other chemokines (MIP-1β and K203 (CCLi3)) following *E. tenella* or *E. maxima* infections (Laurent et al., 2001). Increased IFN-γ levels in response to such infections are well documented both in vitro (Lillehoj and Choi, 1998) and in vivo (Dalloul et al., 2003; Laurent et al., 2001; Min et al., 2003) especially in early response to *E. tenella* infection (Yun et al., 2000), consistent with the present results.

Other cytokine/chemokine elements also show differential expression patterns following treatments with different *Eimeria* species. IL-18, a Th1 type cytokine, was induced at 18 h in *E. acervulina* and *E. tenella* exposed macrophages but only after 48 h in response to *E. maxima* exposure. Also, the CCL chemokine MIP-1β (CCLi1) was observed to peak in expression at 18 h in response to *E. acervulina* and *E. tenella* sporozoites but its maximal induction was at only 4 h in *E. maxima*. Furthermore, although little is known about the chemokine ah221 (CCLi7), it is noteworthy that it was upregulated very early during all three *Eimeria* infections, albeit at a much higher level in *E. acervulina*, and that transcript levels came down progressively with time. While there is an underlying macrophage transcriptional response, which is shared among the *Eimeria* species, unique differences are obvious in the specific elements of the response as well as in the magnitude, direction, and timing of the immune responses to each individual species. In addition, many elements of unknown function were observed to be highly induced or repressed in both the core group and within the distinctive responses to individual *Eimeria* species. Therefore, more questions remain to be answered and investigations are underway to characterize in vivo immune responses using this macrophage array as well as specific mucosal immune responses using a novel 10,000 element intestinal array derived from intraepithelial lymphocytes.

Acknowledgements

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